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Nerve Insensitivity to *cis*-Cypermethrin is Expressed in Adult *Heliothis virescens*

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Abstract: Adult and third-instar larval *Heliothis virescens* were examined for nerve insensitivity to *cis*-cypermethrin using a rapid and robust neurophysiological method. Three laboratory strains, BRC susceptible, PEG-87 resistant and MS2 metabolic resistant, were compared with a series of pyrethroid-resistant strains collected from a field site in Louisiana, USA, during the 1992 cotton season. Nerve insensitivity was present in a proportion of the individuals in both the PEG-87 and the field strains, at both life stages, minimal in the MS2 strain and absent in the BRC strain. Frequencies of nerve-sensitive and nerve-insensitive individuals in each strain were not significantly different at the two life stages examined.

Key words: *Heliothis virescens*, nerve insensitivity, pyrethroids, resistance

1 INTRODUCTION

The tobacco budworm *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) is a major pest of cotton in the USA, Central and South America.[1,2] A key factor in the pest status of *H. virescens* has been its ability to develop resistance to all modern groups of insecticides.[3,4] The synthetic pyrethroids are currently the most widely used insecticides for the control of cotton pests and *H. virescens* has developed resistance to them throughout its geographical range.[5] Since no single alternative group of chemicals is currently available to replace the pyrethroids for large-scale control of *H. virescens*, research to prolong their efficacy has become paramount.

The effective management of pyrethroid resistance in *H. virescens* relies on accurate monitoring of the frequency of resistant individuals and an understanding of the expression of mechanisms of resistance in field populations of the pest.[6] Monitoring of pyrethroid resistance in *H. virescens* in the USA is based on the response of adult pheromone-trapped moths to insecticide-coated glass vials (Adult Vial Test).[7] A fundamental assumption of this easily performed and widely adopted test is that major pyrethroid resistance mechanisms will be equally expressed in both larval and adult stages of *H. virescens*,[6] although the expression of specific resistance mechanisms in the two life stages of the pest was not originally investigated.

Physiological and biochemical mechanisms of resistance to pyrethroids have been widely studied in larval *H. virescens* and include delayed penetration of the insecticide,[8,9] enhanced metabolism by monooxygenase[10,11] and esterase enzymes[12] and nerve insensitivity due to an altered target site.[13,14] Nerve insensitivity appeared to be the principal mechanism of pyrethroid resistance in larval *H. virescens* from US field collections made during the 1990 and 1991 cotton seasons.[15–17]

Nerve-insensitive *H. virescens* have been shown to possess qualitative changes in the neuronal voltage-gated sodium channels[18,19] that are the primary target site for pyrethroid insecticides,[20] allowing the channels to open and close normally in the presence of pyrethroid. Quantitative changes in the number of sodium channels expressed by resistant *Musca domestica* have

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been reported,[21] but the significance of these observations in terms of nerve insensitivity in this insect remains unclear. Despite recent advances in the understanding of the molecular basis for nerve insensitivity and its expression in larval *H. virescens*, the expression of this and other resistance mechanisms in the adult stage of the pest remains poorly understood.

In susceptible nerve-sensitive insects, sodium channels are blocked open by some pyrethroids, causing repetitive firing in the peripheral and central nervous systems.[22,23] The aim of this research was to modify a neurophysiological assay originally designed to detect neuronal responses of larval *H. virescens* to cumulative doses of pyrethroids[14] for use in monitoring the expression of nerve insensitivity in adults.

2 EXPERIMENTAL METHODS

2.1 Insects

Susceptible (BRC) and pyrethroid-resistant (PEG-87) laboratory strains of *H. virescens* were obtained from Zeneca Agrochemicals, Jealott's Hill, UK and Richmond US research centres respectively. The MS2 laboratory strain of *H. virescens* was specifically selected for metabolic resistance by outcrossing of the Dupont and BRC strains (Imong and McCaffery, unpublished). Eggs of field-strain *H. virescens* were randomly collected from cotton fields during June, July and August of the 1992 cotton season from the Red River site in North West Louisiana, dispatched to Reading by express carrier (Clower, pers. comm.) and designated RR1, RR2 and RR3. Insects were reared in the laboratory as described previously.[24] Each generation the PEG-87 and MS2 strains were selected within cypermethrin at first-instar (500 mg litre^{-1} foliar residue) and adult (0·1 μg per moth topical) life stages respectively as described previously.[24] First-instar PEG-87 strain larvae were exposed to cotton leaves that had previously been dipped into aqueous dispersions of a cypermethrin emulsifiable concentrate ('Cymbush'; see Section 2.2). Forty-eight hours after emergence, MS2 strain adults were topically dosed in the left eye with technical grade *cis*-cypermethrin dissolved in acetone. Forty-eight hours after selection survivors from the PEG and MS2 strains were included in experiments or reserved for rearing. BRC strain and all field-strain insects were maintained in the laboratory without exposure to insecticides.

2.2 Chemicals

Technical grade *cis*-cypermethrin (98·8%), was supplied by Shell Research Limited, Analytical Chemistry Division, Sittingbourne, UK in the form of a 46 : 54 mixture of (*S*)-α-cyano/(1*R*,3*R*) and (*R*)-α-cyano/(1*S*,3*S*) isomers. For neurophysiological experiments, *cis*-cypermethrin was suspended in saline; a stock solution of 1 mM *cis*-cypermethrin was prepared in acetone and diluted directly into saline to give a final range of concentrations of 1 to 100 nM. Diagnostic doses of technical grade *cis*-cypermethrin (0·1 μg μl^{-1}) were dissolved in acetone (Analar). A cypermethrin EC ('Cymbush'; *cis* : *trans*-cypermethrin ratio 40 : 60) was supplied by Zeneca Agrochemicals, Jealott's Hill, Bracknell, UK.

2.3 Diagnostic dose bioassays

One microlitre drops of acetone containing 0·1 μg *cis*-cypermethrin were applied to the mesothoracic dorsum of mid-third-instar insects weighing 19–24 mg. Control insects were treated in the same way with acetone alone. Each treatment and control group was made up from four replicates of 10 larvae. Insects were treated in individual 30-ml plastic pots, supplied with a 0·5 cm^3 cube of artificial diet, maintained in an incubator at 25°C and assessed for mortality after 72 h.

2.4 Neurophysiological assay for nerve insensitivity

Neurophysiological assays were carried out in lepidopteran saline[25] at 25(±0·5)°C. The effect of *cis*-cypermethrin on the spontaneous multi-unit activity of nerves from 30–35 adult and third-instar larvae of each strain was measured using the cumulative dose response assay described in detail by McCaffery *et al.*[15] and modified for use with adults. Third-instar larvae (19–24 mg) were decapitated, opened dorso-medially and pinned out on a layer of Sylgard resin (Dow Corning, UK). The inner surface of the body wall and its associated nervous tissue was exposed by dissection and bathed in saline. Adult moths were decapitated and their wings removed before being pinned on a layer of Sylgard resin. A longitudinal dorso-medial incision was made through the thorax and the exposed inner thoracic muscles, body wall and associated nervous system bathed in saline. Measurements of spontaneous neuronal activity were made from nerves in the lateral wall of the thorax.

For both larval and adult preparations, a peripheral nerve was picked up with a 27-gauge stainless steel, suction, recording electrode with an insulated outer coating. A stainless steel entomological pin grounded the preparation and served as a reference electrode. Extracellular neuronal activity was amplified and filtered with a high-gain low-noise front end amplifier and conditioning system (Neurolog Digitimer, UK) before relay to a MacLab 2e data recording and analysis instrument connected to a portable Macintosh com-

puter. Spontaneously occurring action potentials were discriminated from background noise above a visually adjusted threshold, and were counted and recorded by computer in 15-s epochs in blocks of 5-min periods. Nerve preparations were first bathed for 5 min in saline, followed by successive 5-min perfusions of saline containing step-wise increasing doses of *cis*-cypermethrin.

The end-point of the assay was defined as the lowest concentration of *cis*-cypermethrin at which the frequency of action potentials was over five times greater than the mean value during the pre-treatment control period (typically 5–40 Hz). In later assays an additional 5-min cypermethrin-free saline control was performed before recording began.

3 RESULTS

3.1 Diagnostic dose bioassays

The diagnostic dose of 0·1 μg *cis*-cypermethrin per insect represents the approximate LD_{99} of the BRC susceptible strain, established from full bioassay results and calculated by Polo Probit analysis[26] [LD_{99} 0·1 μg per insect; 95% confidence limits 0·038–0·212 μg] (full data not shown). One hundred percent of the PEG and MS2 laboratory strain larvae survived this dose, confirming their status as pyrethroid-resistant strains. The RR1, RR2 and RR3 field-strain larvae gave a more heterogeneous response to the diagnostic dose, with overall survivorship of 58%, 63% and 80% respectively, indicating a progressive increase in the frequency of expression of resistance throughout the cotton season.[17]

3.2 Neurophysiology

The neuronal responses of *H. virescens* larvae and adults to *cis*-cypermethrin generally conformed to a consistent pattern. Susceptible, nerve-sensitive individuals responded to 1 nM or 5 nM *cis*-cypermethrin with a >5-fold rise in the rate of firing of spontaneous action potentials recorded from the nerve. Increases in bursts of nerve firing and repetitive patterns of firing were also noted in cypermethrin-treated nerve-sensitive individuals. Intermediate individuals failed to respond to the lowest concentrations of *cis*-cypermethrin, but responded to 10 nM or 50 nM with the same symptomatic rise in frequency of action potentials. Resistant, nerve-insensitive individuals either failed to respond to all but the highest concentrations of *cis*-cypermethrin (100 nM), or failed to respond to any concentration of *cis*-cypermethrin throughout the assay. Comparisons of the numbers of individual larvae and adults from the BRC, MS2, PEG and RR3 strains responding at each concentration of *cis*-cypermethrin were made with χ^2 tests and are summarised in Table 1 and illustrated in Fig. 1.

Thirty to thirty-five individual larvae and adults from each strain were examined (see Table 1) and the neurophysiological results combined into profiles for each strain and life stage (Fig. 1). Larvae and adults of the BRC susceptible strain all responded at 1 nM or 5 nM

TABLE 1
Neurophysiological Responses to *cis*-cypermethrin in 3rd-Instar and Adult *Heliothis virescens* from the Laboratory and Field (1992 Season)

		Numbers of insects responding in assay at concentration cis-cypermethrin (nM).							
Strain	*Stage*	*1*	*5*	*10*	*50*	*100*	*>100*	χ^2	*df*
BRC	Larvae[b]	24	6	0	0	0	0		
	Adults	21	9	0	0	0	0	0·8	1 ns[a]
PEG	Larvae[b]	7	3	3	4	7	6		
	Adults	5	2	2	7	6	8	1·91	5 ns[a]
MS2	Larvae	14	13	3	0	0	1		
	Adults	17	7	2	2	1	1	5·28	5 ns[a]
1992 field strains									
RR1	Larvae[b]	9	9	4	2	4	5		
	Adults	7	10	2	3	4	4	1·14	5 ns[a]
RR2	Larvae[b]	7	9	2	4	5	6		
	Adults	8	6	5	2	5	4	2·88	5 ns[a]
RR3	Larvae[b]	7	5	3	1	4	11		
	Adults	4	5	1	2	6	12	2·57	5 ns[a]

[a] χ^2 values for comparisons between larvae and adults in each strain are not significantly different even though expected values of <5 normally bias χ^2 towards significant differences.
[b] 1992 larval data from McCaffery, Holloway and Gladwell.[17]

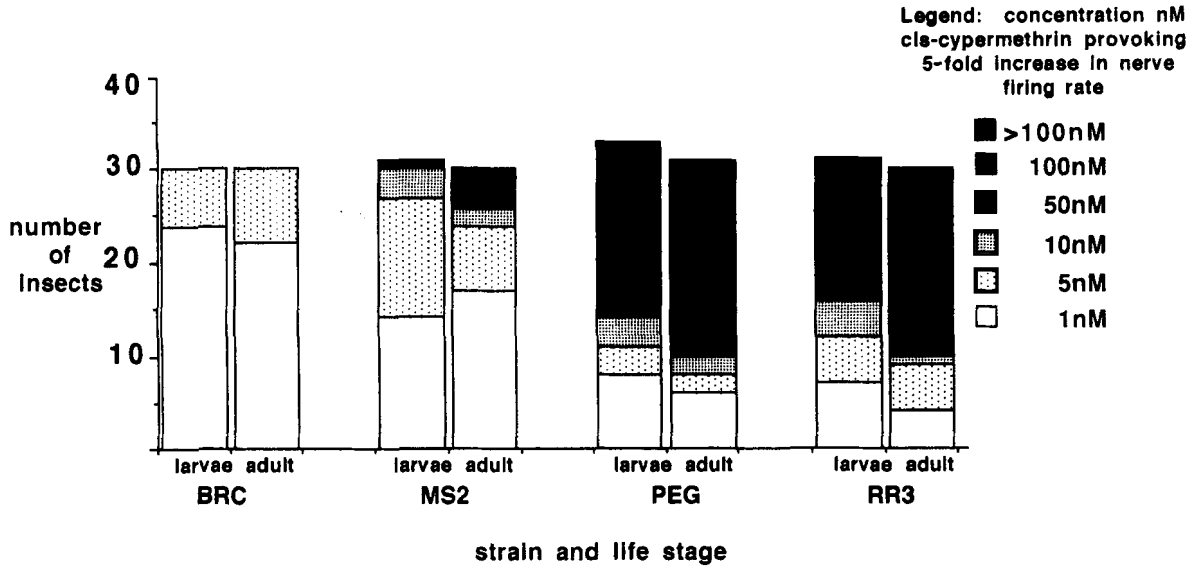

Fig. 1. The phenotypic distribution of neuronal responses to cumulative doses of *cis*-cypermethrin in 3rd-instar larvae (first of each series) and adults (second of each series) from laboratory and field strains of *Heliothis virescens*.

cis-cypermethrin, giving a typical nerve-sensitive profile. Neuronal responses of larvae and adults of the MS2 strain insects were not significantly different from those of the nerve-sensitive BRC susceptible strain at comparative life stages (larvae $\chi^2 = 9{\cdot}2$, 3df, adults $\chi^2 = 6{\cdot}7$, 5df), with the majority of insects responding at 1 nM or 5 nM *cis*-cypermethrin, despite the fact that all MS2 strain larvae survived the diagnostic dose of 0·1 μg *cis*-cypermethrin. Larvae and adults of the PEG-87 resistant strain showed a range of responses, with every type of response including nerve-sensitive (1 nM–5 nM), intermediate (10 nM–50 nM) and nerve-insensitive (100 and >100 nM) individuals.

The RR1, RR2 and RR3 field-strain profiles show a range of responses similar to those of the PEG-87 strain (Fig. 1). The frequency of expression of nerve insensitivity increased in adults of the later Red River strains (Fig. 2), a trend of increasing expression already noted in larval collections from this site.[15] For all strains the neurophysiological profiles were not significantly different at the two life stages examined.

4 DISCUSSION

Whilst nerve insensitivity has been shown to be a major mechanism of pyrethroid resistance in larval stages of *H. virescens*,[15–17] mechanisms of pyrethroid resistance in the adult are poorly understood.[5] The occurrence of nerve insensitivity to pyrethroids has been reported in the absence of direct neurophysiological studies on the

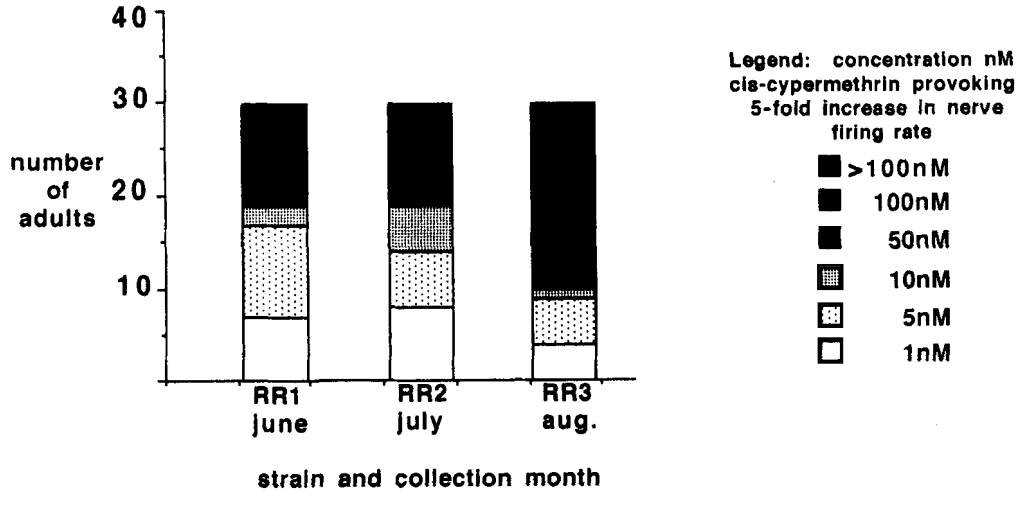

Fig. 2. The phenotypic distribution of neuronal responses to cumulative doses of *cis*-cypermethrin in adult *Heliothis virescens* from field strains collected during June, July and August of the 1992 cotton season from the Red River field station, Louisiana USA showing progressive expression of nerve insensitivity.

basis that pyrethroid toxicity is unaffected by metabolic synergists, that tests for metabolic mechanisms of resistance fail, that resistant insects contain high titres of unmetabolised toxin at the target site,[27] that cross-resistance is reported to other pyrethroids and DDT, and that behavioural symptoms of toxicology are not apparent in an initial post-treatment period.[28] Although each of these observations can be taken as indirect evidence of nerve-insensitivity resistance to pyrethroids, they are not in themselves sufficient to demonstrate the presence of this resistance mechanism.[29] A more direct approach is to study the effect of the toxin on nerve preparations of the insect which by-pass behavioural, penetration and metabolism resistance factors.

The cumulative dose–response assay reported here was originally designed with the aim of distinguishing nerve-sensitive individuals from those expressing nerve insensitivity to pyrethroids[14] and has recently been used in the field to distinguish resistance phenotypes of *Helicoverpa armigera* (Hubner) in India (McCaffery *et al.*, unpublished). Levels of nerve tolerance are interpreted relative to the BRC susceptible strain and in the present study neuronal tolerance of >5 nM is arbitrarily described as insensitive because this level of tolerance is greater than that found in the susceptible laboratory strain. Using a 1 nM–100 nM range of *cis*-cypermethrin concentrations, the current neurophysiological assay clearly detects the expression of high levels of nerve insensitivity. The close correlation between larval survivorship in diagnostic dose assays and neurophysiological responses as previously reported[17] suggests that increases in nerve tolerance detected with the current assay are associated with pyrethroid resistance.

The MS2 laboratory strain is nerve-sensitive and toxicologically resistant (survivorship of discriminating dose 100% MS2). A possible explanation for this apparent anomaly is that MS2 insects are sufficiently protected by other resistance mechanisms (particularly increases in metabolism and elimination) so reliance on target-site insensitivity is reduced. The multi-factorial resistant PEG-87 strain is known to be highly metabolically resistant, largely due to the presence of a powerful monooxygenase system,[10,11] and McCaffery[30] has suggested that, under continued pyrethroid selection pressure, metabolic resistance mechanisms superseded nerve insensitivity, leading to its deselection. Recent reports suggest that metabolic resistance mechanisms may now be expressed in field populations of *H. virescens*.[31] The heterogeneity of expression of nerve insensitivity in the PEG-87 strain reported here appears to support the hypothesis that at least some of the PEG-87 strain insects are similar to those of the MS2 strain and can survive pyrethroid poisoning without the concomitant expression of high levels of nerve insensitivity.

Alternatively, a low level of nerve insensitivity may be expressed by the PEG-87 and MS2 strain insects at a level below the limits of detection using a five-step, 1–100 nM *cis*-cypermethrin concentration range with the current assay. Wilkinson[27] found that high titres of cypermethrin could be detected in the nerves of resistant PEG-87 larvae within the first 4 h of dosing, even though delayed penetration,[8] increased metabolism,[10,11] and increased elimination[8] are all expressed by larvae of the PEG-87 strain. Since fewer than 1% of sodium channels need to be affected by pyrethroid before symptoms of intoxication and fatal lesions occur,[20] it was suggested that a degree of nerve insensitivity is a pre-requisite of pyrethroid survival before metabolic resistance mechanisms can reduce pyrethroid titres at the target site. The existence of lesser degrees of nerve insensitivity in the predominantly metabolically resistant PEG-87 and MS2 strains as suggested by Wilkinson[27] is speculative but may involve co-expression with metabolic resistance.

Adults from the three Red River field collections showed increasing expression of nerve insensitivity throughout the cotton season (from 45% in June to 48% in July and 61% in August) as shown in Fig. 2. A likely explanation for this trend is that continued pyrethroid use in the field exerts sustained selection pressure at the target site[17] and that the expression of nerve insensitivity in larvae is carried through metamorphosis into the adult life stage. The highest levels of nerve insensitivity recorded in this study were detected in the RR3 field strain, in which 61% of larvae and 70% of adults failed to respond to 1 nM and 5 nM *cis*-cypermethrin; clearly the expression of nerve insensitivity can be widespread in field-collected adults.

A basic assumption of the US adult vial test is that the balance of expression of pyrethroid resistance mechanisms is equal in all life stages of the pest, the adult as well as the larva. Toxicological data appear to support this. Early studies found a good correlation between overall levels of resistance determined by larval and adult assays.[32] Synergism studies have revealed a piperonyl butoxide (PBO) suppressible metabolic resistance mechanism expressed in both larval and adult stages of the pest,[33,34] albeit with higher levels of PBO synergism in the larval stage than in the adult stage.[33] In-vitro studies of model substrate metabolism by both larval and adult preparations have revealed a good correlation between the expression of monooxygenase enzymes in the two life stages, although expression is higher in the larval stage.[35]

Metabolic resistance mechanisms may be poorly expressed in the adult stage of this pest accounting at least in part for the reduction in pyrethroid tolerance of *H. virescens* adults compared with larvae,[33] and the decline of pyrethroid tolerance reported during the adult life stage of the related pest *Helicoverpa armigera*[36] where pyrethroid resistance is thought to be predominantly caused by metabolic mechanisms.[37] Interestingly, in the US, where nerve insensitivity has been the primary pyrethroid resistance mechanism, no

decline in survival at the discriminating dose was observed in adult *H. virescens* up to eight days post-treatment.[36] Studies of in-vitro metabolism of *cis*-[^{14}C] cypermethrin in both larval and adult life stages of the BRC and MS2 laboratory strains support the hypothesis that the balance of expression of metabolic resistance mechanisms may be biased towards the larval stage (Holloway and McCaffery, unpublished).

The results described here and those in our preliminary study[38] suggest that high levels of nerve insensitivity to *cis*-cypermethrin can be expressed not only by larval stages of *H. virescens* but also by adults. Reduced neuronal sensitivity to allethrin has also been reported to be expressed in larval and adult *H. virescens*,[39] although, interestingly, expression was higher in adults than in larvae of comparable strains. Further studies will be required to establish whether the correlation between expression of larval and adult nerve insensitivity to cypermethrin reported here extends to other pyrethroids. If this is the case, bioassays involving adults or larvae should be equally effective for monitoring pyrethroid resistance when nerve insensitivity is the predominant resistance mechanism in the field. The expression of nerve insensitivity by both life stages of *H. virescens* suggests that this mechanism can confer protection from pyrethroid poisoning throughout the life of the insect.

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